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(57) Abstract

Disclosed is a composition comprising at least two synthetic, cooperative oligonucleotides, each comprising a region complementary to one of tandem, non-overlapping regions of a target single-stranded nucleic acid, and each further comprising a dimerization domain at a terminus of each of the oligonucleotides, the dimerization domains of the oligonucleotides being complementary to each other. Also disclosed are duplex structures, ternary complexes, pharmaceutical formulations, and methods utilizing the cooperative oligonucleotides of the invention.

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COOPERATIVE OLIGONUCLEOTIDES

BACKGROUND OF THE INVENTION

Progress in chemical synthesis of nuclease resistant oligonucleotides (*Methods Mol. Biol.* (1993) Vol. 20, (Agrawal, ed.) Humana Press, Totowa, NJ) and developments in large scale solid phase synthesis of oligonucleotides ((Agrawal, ed.) *Methods Mol. Biol.* (1993) Vol. 20, Humana Press, Totowa, NJ); Padmapriya et al. (1994) *Antisense Res. Dev.* 4:185-199) has permitted antisense oligonucleotides to advance to human clinical trials (Bayever et al. (1993) *Antisense Res. Dev.* 3:383-390). In principle, antisense oligonucleotides utilize highly sequence-specific complementary nucleo-base recognition of target nucleic acids through Watson-Crick hydrogen bonding between A and T, and G and C, that leads to the development of less toxic and more site specific chemotherapeutic agents (Stephenson et al. (1978) *Proc. Natl. Acad. Sci. (USA)* 75:285-288). As per theoretical calculations, an oligonucleotide of 13 or more bases long should bind to a unique sequence that occurs only once in a eucaryotic mRNA pool.

Contrary to the popular belief, it was recently shown that the increase in the length of an antisense oligonucleotide beyond the minimum length that can hybridize to the target (i.e. 11-14 bases) decreases its specificity rather than increasing (Woolf et al. (1992) *Proc. Natl. Acad. Sci. (USA)* 89:7305-7309). Potentially, this decrease in hybridization specificity would lead to non-sequence-specific

target binding and subsequent increased toxicity (Stein et al. (1993) *Science* 261:1004-1012).

Thus, what is needed are improved antisense oligonucleotides optimized for therapeutic and diagnostic use which have improved affinity, specificity, and biological activity, and little or no toxicity.

SUMMARY OF THE INVENTION

The present invention provides cooperative oligonucleotides with improved sequence specificity for a single-stranded target, reduced toxicity, and improved biological activity as antisense molecules.

Surprisingly, it has been discovered that two short oligonucleotides (25 nucleotides or less) bind to adjacent sites on the target nucleic acid in a cooperative manner, allowing for an interaction with greater sequence specificity than can a single longer oligonucleotide having a length equal to the two shorter oligonucleotides.

Accordingly, in a first aspect, the present invention provides a composition including at least two synthetic cooperative oligonucleotides, each comprising a region complementary to one of tandem, non-overlapping regions of a target single-stranded nucleic acid, and a dimerization domain at a terminus of each of the oligonucleotides. The dimerization domains of the cooperative oligonucleotides are complementary to each other,

and the target nucleic acid being an mRNA, single-stranded viral DNA, or single-stranded viral RNA.

In some preferred embodiments, the oligonucleotides each are complementary to tandem regions of the target nucleic acid that are separated by 0 to 3 bases. In some preferred embodiments, each of the oligonucleotides are about 9 to 25 nucleotides in length.

In one embodiment, the composition consists of two cooperative oligonucleotides, the dimerization domain of a first or one of the oligonucleotides being located at its 3' terminal portion, and being complementary to the dimerization domain of a second or the other oligonucleotide which is located at its 5' terminal portion. Alternatively, the dimerization domain of the first cooperative oligonucleotide is located at its 3' terminal portion, and is complementary to the dimerization domain of a second oligonucleotide which is located at its 3' terminal portion. Alternatively, the dimerization domain of the first cooperative oligonucleotide is located at its 5' terminal portion, and is complementary to a dimerization domain of the second oligonucleotide which is located at its 5' terminal portion.

The invention provides in another aspect a duplex structure comprising first and second synthetic cooperative oligonucleotides, each oligonucleotide comprising a region complementary to the non-overlapping, tandem regions of the target

nucleic acid which is an mRNA, single-stranded viral RNA, or single-stranded viral DNA.

The first oligonucleotide in the duplex has a terminal dimerization domain complementary and hybridized to the dimerization domain of the second oligonucleotide. In some embodiments, each of the oligonucleotides are about 9 to 25 nucleotides in length, and in others, the dimerization domains of the first and second oligonucleotides each comprise about 3 to 7 nucleotides. In some embodiments, the invention provides first and second oligonucleotides which are complementary to tandem regions of the target nucleic acid separated by 0 to 3 bases. In another embodiment, the duplex structure is hybridized to the target nucleic acid.

The invention also provides pharmaceutical formulations containing the compositions or duplex structures described above, and methods of inhibiting the expression of a nucleic acid *in vitro* comprising the step of treating the nucleic acid with the pharmaceutical formulations of the invention. In some embodiments, the first and second oligonucleotides are complementary to an HIV DNA or an HIV RNA.

In another aspect, the invention provides a ternary structure comprising a first synthetic cooperative oligonucleotide, a second synthetic cooperative oligonucleotide, and a third synthetic cooperative oligonucleotide, each oligonucleotide comprising a region complementary to one of tandem, non-overlapping regions of a target nucleic acid, and each comprising a dimerization domain at one or

both of their termini. The dimerization domain of the first oligonucleotide is complementary and hybridized to a first dimerization domain at one terminus of the third oligonucleotide and the dimerization domain of the second oligonucleotide is complementary and hybridized to a second dimerization domain at the other terminus of the third oligonucleotide when the first, second, and third oligonucleotides are hybridized to the target nucleic acid. The target nucleic acid is an mRNA, a single-stranded viral RNA, or a single-stranded viral DNA. In some embodiments, the ternary structure is hybridized to the target nucleic acid.

In another embodiment, the invention provides methods of inhibiting the expression of a nucleic acid comprising the step of contacting the nucleic acid with at least one cooperative oligonucleotide of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1A is a schematic representation of the cooperative binding of two short oligonucleotides to tandem sites;

FIG. 1B is a schematic representation of the binding to adjacent sites on a target nucleic acid of cooperative oligonucleotides that have extended antisense dimerization domains and their dimerization;

FIG. 1C is a schematic representation of the binding of three cooperative oligonucleotides of the invention to adjacent sites on a target nucleic acid;

FIG. 2A is a graphic representation showing the thermal melting profile (dA/dT vs. T) of oligonucleotides 1-7 shown in Table 2 with their DNA target;

FIG. 2B is a graphic representation showing the thermal melting profile (dA/dT vs. T) of oligonucleotides 1+2, 1+3, 1+4, and 5 shown in Table 2 with their DNA target;

FIG. 3 is a graphic representation showing the thermal melting profiles (dA/dT vs. T) of the oligonucleotide combinations with extended antisense dimerization domains (10+14, 11+15, 9+14, 12+16, and 13+17);

FIG. 4A is an autoradiogram showing the RNase H hydrolysis pattern of the RNA target sequence in the presence of oligonucleotides 5, 1, 2, 1+2, 14, 10, and 10+14 at different time points;

FIG. 4B is an autoradiogram showing the RNase H hydrolysis pattern of the RNA target sequence in the presence of oligonucleotides 5, 13, 17, and 13+17 at different time points;

FIG. 5 is an autoradiogram showing the RNase H hydrolysis pattern of RNA target in the presence of the mismatched oligonucleotides 23, 24, 18 and 19 compared to the control matched oligonucleotide 5 and 1 at different time points;

FIG. 6 is a graphic representation showing the ability of cooperative oligonucleotide oligonucleotides 1+2 (--◊--), and 13+17 (--o--), and control oligonucleotides 5 (--□--) and 20 (--Δ--) at varying concentrations to inhibit HIV-1 in a cell culture system;

FIG. 7 is a graphic representation showing the percent inhibition of HIV-1 in cell cultures by cooperative antisense oligonucleotides 1+2, 13+17, 9+14, 10+14, and 12+16 and by control antisense oligonucleotides 5 and 20, present at two different concentrations; and

FIG. 8 is a graphic representation showing the relationship between meeting temperature (Tm) and percent HIV-1 inhibition for cooperative oligonucleotides 10+14, 12+16, and 13+17.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Cooperative interactions between biological macromolecules are important in nature. For example, the cooperative interactions between proteins and nucleic acids are vital for the regulation of gene expression. Cooperative interactions serve to improve sequence specificity, affinity, and biological activity (Ptashne (1986) *A Genetic Switch*; Blackwell Scientific Publications and Cell Press: Palo Alto, CA). Cooperative binding of drugs to DNA (Asseline et al. (1984) *Proc. Natl. Acad. Sci. (USA)* 81:3297-3301; Rao et al. (1991) *J. Org. Chem.* 56:786-797), of oligonucleotides or their conjugates to single stranded DNA (Tazawa et al. (1972) *J. Mol. Biol.* 66:115-130; Maher et al. (1988) *Nucl. Acids Res.* 16:3341-3358; Springgate et al. (1973) *Biopolymers* 12:2241-2260; and Gryaznov et al. (1993) *Nucl. Acids Res.* 21:5909-5915), of oligonucleotides to RNA (Maher III et al. (1987) *Arch. Biochem. Biophy.* 253:214-220), and of oligonucleotides to double- stranded DNA through triplex formation (Strobel et al. (1989) *J. Am. Chem. Soc.* 111:7286-7287; Distefano et al. (1991) *J. Am. Chem. Soc.* 113:5901-5902; Distefano et al. (1992) *J. Am. Chem. Soc.* 114:11006-11007; Colocci et al. (1993) *J. Am. Chem. Soc.* 115:4468-4473; Colocci et al. (1994) *J. Am. Chem. Soc.* 116:785-786) has been documented. Although these studies demonstrated the advantages of using cooperative interactions for small molecule-based drug development, there are no reports of optimizing the design of cooperative oligonucleotides for therapeutic uses.

The present invention provides synthetic oligonucleotides which interact with mRNA, single-stranded viral RNA, or single-stranded viral DNA ("target nucleic acids"), and have improved affinity, specificity, and biological activity as antisense molecules. At least two of the oligonucleotides of the invention are used to interact with a target nucleic acid, thereby enabling them to interact cooperatively, synergistically enhancing their ability (singly) to inhibit expression of the target nucleic acid.

The term "synthetic oligonucleotide" for purposes of this invention includes chemically synthesized polymers of about 7 to about 25, and preferably from about 9 to about 23 nucleotide monomers (nucleotide bases) connected together or linked by at least one 5' to 3' internucleotide linkage.

Some cooperative oligonucleotides of the invention are complementary to non-overlapping, tandem regions of the target nucleic acid, as shown in FIG. 1A, while others are complementary to adjacent sites (FIGS. 1B and 1C). At least two of these oligonucleotides can used to control target nucleic acid expression.

For purposes of the invention, the term "oligonucleotide complementary to a target nucleic acid" is intended to mean an oligonucleotide sequence that binds to the nucleic acid sequence under physiological conditions, e.g., by Watson-Crick base pairing (interaction between oligonucleotide and single-stranded nucleic acid in

antiparallel orientation) or by Hoogsteen base pairing (interaction between oligonucleotide and double-stranded or single-stranded nucleic acid in parallel orientation) or by any other means including in the case of a oligonucleotide binding to RNA, pseudoknot formation. Such binding (by Watson-Crick base pairing) under physiological conditions is measured as a practical matter by observing interference with the function of the nucleic acid sequence.

The inhibitory ability of the cooperative oligonucleotides of the invention is enhanced even further when these oligonucleotides also include a terminal portion (i.e., a "dimerization domain") which is not complementary to the target nucleic acid, but rather which is complementary to each other, thereby enabling the formation of a dimers (FIG. 1B). The interaction of these cooperative oligonucleotides with the target nucleic acid leads to the formation of a more stable ternary complex as the result of dimerization of the complementary dimerization domains of these oligonucleotides. When the cooperative oligonucleotides of the invention have dimerization domains and hybridize together to form a duplex, the regions of the cooperative oligonucleotides which are complementary to the target nucleic acid may be separated by 0 to 3 bases.

The cooperative oligonucleotides of the invention may have any nucleotide sequence, as long as a portion of its sequence is complementary to a portion of a target nucleic acid. Any nucleic acid

may be targeted by the cooperative oligonucleotides of the invention including viral, bacterial, and cellular genes, mRNAs, or cDNAs. Further, the terminal dimerization domains of cooperative oligonucleotides which form duplexes with each other may not be not complementary to the target nucleic acid. These dimerization domains may be at the 3' termini of both cooperative oligonucleotides, at the 5' termini of both cooperative oligonucleotides, or at the 3' terminus of one cooperative oligonucleotide and the 5' terminus of the other cooperative oligonucleotide.

The cooperative oligonucleotides of the invention are composed of deoxyribonucleotides, ribonucleotides, or any combination thereof, with the 5' end of one nucleotide and the 3' end of another nucleotide being covalently linked, in some cases, via a phosphodiester internucleotide linkage. The oligonucleotides can be prepared by art recognized methods such as phosphoramidate, H-phosphonate chemistry, or methylphosphoramidate chemistry (*see, e.g.*, Uhlmann et al. (1990) *Chem. Rev.* 90:543-584; Agrawal et al. (1987) *Tetrahedron. Lett.* 28:(31):3539-3542); Caruthers et al. (1987) *Meth. Enzymol.* 154:287-313; U.S. Patent 5,149,798) which can be carried out manually or by an automated synthesizer and then processed (reviewed in Agrawal et al. (1992) *Trends Biotechnol.* 10:152-158).

The oligonucleotides of the invention may also be modified in a number of ways, for example, to enhance stability, without compromising their

ability to hybridize to nucleotide sequences contained within a targeted region of a particular gene.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which at least two of its nucleotides are covalently linked via a synthetic linkage, i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide in which the 5' nucleotide phosphate has been replaced with any number of chemical groups. For example, oligonucleotides with phosphorothioate linkages can be prepared using methods well known in the field such as methoxyphosphoramidite (*see, e.g.*, Agrawal et al. (1988) *Proc. Natl. Acad. Sci.* (USA) 85:7079-7083) or H-phosphonate (*see, e.g.*, Froehler (1986) *Tetrahedron Lett.* 27:5575-5578) chemistry. The synthetic methods described in Bergot et al. (*J. Chromatog.* (1992) 559:35-42) can also be used. Examples of other chemical groups include alkylphosphonates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphoramidites, phosphate esters, 2-O-methyls, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters, and thiono triesters. Any of these chemical groups or linkages may also be substituted with various other chemical groups, e.g., an aminoalkylphosphonate. Oligonucleotides with these chemical groups can be prepared according to known methods (see, e.g., Agrawal and Goodchild (*Tetrahedron Lett.* (1987) 28:3539-3542); Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1988) 85:7079-7083); Uhlmann

et al. (*Chem. Rev.* (1990) 90:534-583; and Agrawal et al. (*Trends Biotechnol.* (1992) 10:152-158). U.S. Patent Application Ser. No. (47508-559), filed on August 9, 1995 discloses "inverted" chimeric oligonucleotides comprising one or more nonionic oligonucleotide region (e.g. alkylphosphonate and/or phosphoramidate and/or phosphotriester internucleoside linkage) flanked by one or more region of oligonucleotide phosphorothioate. The phosphorothioate linkages may be mixed Rp and Sp enantiomers, or they may be stereoregular or substantially stereoregular in either Rp or Sp form (see Iyer et al. (1995) *Tetrahedron Asymmetry* 6:1051-1054). Thiono triesters can be prepared according to the methods described in U.S. Ser. No. 08/409,169, filed March 23, 1993. Oligonucleotides with phosphorothioate linkages can be prepared using methods well known in the field such as phosphoramidite (see, e.g., Agrawal et al. (1988) *Proc. Natl. Acad. Sci. (USA)* 85:7079-7083). or by H-phosphonate (see, eg., Froehler (1986) *Tetrahedron Lett.* 27:5575-5578) chemistry. The synthetic methods described in Bergot et al. (*J. Chromatog.* (1992) 559:35-42) can also be used.

Oligonucleotides which are self-stabilized are also considered to be modified oligonucleotides useful in the methods of the invention (Tang et al. (1993) *Nucleic Acids Res.* 20:2729-2735). These oligonucleotides comprise two regions: a target hybridizing region; and a self-complementary region having an oligonucleotide sequence complementary to a nucleic acid sequence that is within the self-stabilized oligonucleotide.

Other modifications include those which are internal or are at the end(s) of the oligonucleotide molecule and include additions to the molecule of the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the viral genome. Examples of such modified oligonucleotides include oligonucleotides with a modified base and/or sugar such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position).

Yet other examples of modifications to sugars include modifications to the 2' position of the ribose moiety which include but are not limited to 2'-O-substituted with an -O- lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl, or allyl group having 2-6 carbon atoms wherein such -O-alkyl, aryl or allyl group may be unsubstituted or may be substituted, (e.g., with halo, hydroxy, trifluoromethyl cyano, nitro acyl acyloxy, alkoxy, carboxy, carbalkoxyl, or amino groups), or with an amino, or halo group. None of these substitutions are intended to exclude the native 2'-hydroxyl group in the case of ribose or 2'-H- in the case of deoxyribose. PCT Publication No. WO 94/02498 discloses traditional

hybrid oligonucleotides having regions of 2'-O-substituted ribonucleotides flanking a DNA core region. U.S. Patent Application Serial No. 08/516,454, filed August 17, 1995, discloses an "inverted" hybrid oligonucleotide which includes an oligonucleotide comprising a 2'-O-substituted (or 2' OH, unsubstituted) RNA region which is in between two oligodeoxyribonucleotide regions, a structure that "inverted relative to the "traditional" hybrid oligonucleotides.

Other modified oligonucleotides are capped with a nuclease resistance-conferring bulky substituent at their 3' and/or 5' end(s), or have a substitution in one nonbridging oxygen per nucleotide. Such modifications can be at some or all of the internucleoside linkages, as well as at either or both ends of the oligonucleotide and/or in the interior of the molecule (reviewed in Agrawal et al. (1992) *Trends Biotechnol.* 10:152-158).

To demonstrate the cooperative nature of the oligonucleotides of the invention, oligonucleotides were prepared as described above and tested for their ability to inhibit the expression of a target gene.

The target chosen was a sequence in the initiation codon region of *gag* mRNA of HIV-1 (SEQ ID NOS:21 and 22) (Agrawal and Tang (1992) *Antisense Res. Dev.* 2:261). A nonlimiting list of some representative *gag* oligonucleotides are shown in TABLE 1.

TABLE 1

SEQ ID NO:	Sequence 3' → 5'	Length (# bases)
21	CTAGAAGGAGAGAGATGGGTGCGAGAG	Target
22	AGAAGGAGAGAGAUGGGUGCGAGAGCGUCAGUAUUAAGC	Target
25	GGAGGCUAGAAGGAGAGAGAUGGGUGCGAGAGCGU	Target
1	CCCACGCTC	9
2	TTCCTCTCTCTA	12
3	CTTCCTCTCTCT	12
4	TCTTCCTCTCTC	12
5	TTCCTCTCTCTACCCACGCTC	21
6	CTTCCTCTCTCTGCCCACGCTC	22
7	TCTTCCTCTCTCCGCCCACGCTC	23
8	CTTCCTCTCTCTA	13
9	TTCCTCTCTCTA G G C	15 15
10	CTTCCTCTCTCT G G C	15
11	CTTCCTCTCTCT G G C C	16

TABLE 1 (con't)

SEQ ID NO:	Sequence (3' → 5'):	Length (# bases)
12	CTTCCTCTCTCT G G C C G	17
57	CTTCCTCTCTCT	12
19	G G C C G C G	7
14	CCCACGCTC C C G	12
15	CCCACGCTC C C G G	13
16	CCCACGCTC C C G G C	14
17	CCCACGCTC C C G G C G C	16
18	CCCACTCTC	9
19	CCAACTCTC	9
20	TCTTCCTCTCTCTACCCACGCTCTC	25
23	TTCCTCTCTCTACCCACTCTC	21
24	TTCCTCTCTCTACCAACTCTC	21
26	TCTTCCTCT T C A C C A G	16

TABLE 1 (con't)

SEQ ID NO:	Sequence* (3' → 5')	Length (# bases)
27	CTCTACC A G G A T G G T G A T A C G	21
28	CACGCTCTC C T C A C T C	16
29	TCTTCCTCT T C A C C	14
30	CTCTACC A G G A T G G T G A	17
31	CACGCTCTC C T C A C	14
32	TCTTCCTCT T C A	12
33	CTCTACC A G G A T G	13
34	CACGCTCTC C T C	12
35	TCTTCCTCT	9

TABLE 1 (con't)

SEQ ID NO:	Sequence[b] (3' → 5')	Length (# bases)
36	CTCTACC	7
37	CACGCTCTC	9

[a] underlined bases represent mismatches
[b] sequence is 5' → 3'

Oligonucleotides 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2) are designed to bind to 21 bases of the target nucleic acid at adjacent sites without any base gap between them (see FIG. 1A and TABLE 1). Thus, contact is expected to be maintained through the 3'-end of the oligonucleotide 1 and the 5'-end of the oligonucleotide 2 when these oligonucleotides bind to the target sequence at the adjacent sites. This results in cooperativity in the interactions of these two oligonucleotides. Oligonucleotides 3 (SEQ ID NO:3) and 4 (SEQ ID NO:4) bind to the same site as oligonucleotide 2 but are separated by 1 and 2 bases on the target sequence, gaps, respectively, from the binding site of oligonucleotide 1. Because of this gap these oligonucleotides are expected not to show any cooperativity in the binding of these oligonucleotide pairs to the target. Oligonucleotide 5 (SEQ ID NO:5) binds to the same 21 base target sequence on the target oligonucleotide that oligonucleotides 1 and 2 together bind. Oligonucleotide 6, a 22mer (SEQ ID NO:6) and oligonucleotide 7, a 23mer (SEQ ID NO:7) have 1 and 2 mismatches, respectively, in position that correspond to 1 and 2 base separation when

oligonucleotides 1+3 and 1+4 bind to the target sequence together. Oligonucleotide 8 (SEQ ID NO:8) is a 13mer control oligonucleotide that binds to the same sequence as oligonucleotides 2 and 3 adjacent to oligonucleotide 1 without a base separation between them.

To further improve the cooperative interactions of the oligonucleotides binding to the target sequence at abutting sites, oligonucleotides 1 and 2 were both extended at the site of junction with complementary sequences so that they form a duplex stem upon interaction with the target, as shown in FIG. 1B. This extended antisense dimerization domain is designed not to have any complementarity with the adjacent bases of the antisense oligonucleotide binding site on the target. Oligonucleotides 9-17 (SEQ ID NOS:9-17) have an extended sequence on either the 5'- or 3'-end of the binding sequence, which forms a duplex stem between the two oligonucleotides when they bind to adjacent sites on the target (FIG. 1B). This extended antisense dimerization domain has no complementarity with the target sequence. Oligonucleotides 9 and 14 form a 3 base pair stem. Oligonucleotides 10 and 14 have the same length of extended antisense dimerization domain but with one base separating the two target sites of the binding oligonucleotide pair. Oligonucleotide pairs 11+15, 12+16, and 13+17 bind to the same length of the sequence on the target as oligonucleotide pair 10+14 but with 4, 5, and 7 base pair extended antisense dimerization domains, respectively.

The initial evidence for cooperative binding of oligonucleotides 1 and 2 to their target sequence comes from thermal melting studies. TABLE 2 shows thermal melting data of the duplexes of these oligonucleotides individually and together with other corresponding oligonucleotides (FIG. 2). When oligonucleotides 1 and 2 bound side by side to the target, the resulting duplex has a Tm of 47.8°C. Duplexes of oligonucleotides 1+3 and 1+4 with the target sequence have Tms of 44.4°C and 46°C, respectively. The oligonucleotides 1 and 3 bind to the target with a 1 base gap between them, and the oligonucleotides 1 and 4 bind to the target with a 2 base gap between them. The Tm of the duplex formed by oligonucleotides 1 and 2 together with the target is more than the average of the duplexes formed by 1 and 2 individually with the target sequence
(TABLE 2).

TABLE 2

Oligos SEQ ID (NO:	Complex[a,b]	Tm, °C
1	CTAGAAGGAGAGAGATGGGTGCGAGAG CCCACGCTC	49.1
2	CTAGAAGGAGAGAGATGGGTGCGAGAG TTCCTCTCTCTA	43.4
3	CTAGAAGGAGAGAGATGGGTGCGAGAG CTTCCTCTCTCT	43.6
4	CTAGAAGGAGAGAGATGGGTGCGAGAG TCTTCCTCTCTC	45.0
5	CTAGAAGGAGAGAGATGGGTGCGAGAG TTCCTCTCTCTACCCACGCTC	67.7
6	CTAGAAGGAGAGAGATGGGTGCGAGAG CTTCCTCTCTCTGCCCACGCTC	64.2
7	CTAGAAGGAGAGAGATGGGTGCGAGAG TCTTCCTCTCTCCGCCCACGCTC	59.9
1+2	CTAGAAGGAGAGAGATGGGTGCGAGAG TTCCTCTCTCTA*CCCACGCTC*	47.8
1+3	CTAGAAGGAGAGAGATGGGTGCGAGAG CTTCCTCTCTCT CCCACGCTC	44.4
1+4	CTAGAAGGAGAGAGATGGGTGCGAGAG TCTTCCTCTCTC CCCACGCTC	45.9
1+8	CTAGAAGGAGAGAGATGGGTGCGAGAG CTTCCTCTCTCTA*CCCACGCTC*	50.5

[a] = underlined bases represent mismatches
[b] = Target (SEQ ID NO:21) is bolded and is 5' → 3'; cooperative oligonucleotides are 3' → 5'.

In contrast, in the latter two cases (1+3 and 1+4), the Tms are below the average of the two individual oligonucleotides in experiment. Further, in the case of the duplex formed with oligonucleotides 1+2 a sharp, single, cooperative transition was noticed (FIG. 2B). However, in the cases of the duplexes formed with 1+3 and 1+4, melting transitions were broad (FIG. 2B). This indicates that the two short oligonucleotides 1 and 2 targeted to two adjacent sites bind in a cooperative fashion, whereas those which bind leaving a one or two base gap between them do not interact cooperatively.

The duplex of oligonucleotide 5 which binds to the entire 21 base length has a Tm of 67.7°C. The duplex of oligonucleotide 6 (SEQ ID NO:6), a 22-mer with a mismatch in place that corresponds to one base gap between oligonucleotides 1 and 3, has a Tm of 64.2°C. Similarly, the duplex of oligonucleotide 7 (SEQ ID NO:7), a 23mer with two mismatches in a position that corresponds to the two base gap between oligonucleotides 1 and 4, has a Tm of 59.9°C. The lower melting temperatures of oligonucleotides 6 and 7 which bind to the target with one or two base mismatches indicate that these oligonucleotides can bind to a number of sites other than the perfectly matched target site at physiological temperatures. Thus, sequence specificity is decreasing.

Thermal melting studies of the duplexes of the oligonucleotides 9-17 demonstrates that the binding of these tandem oligonucleotides is further

facilitated by the duplex stem (i.e., antisense dimerization domain) formed by extending the antisense dimerization domain. The stability of the ternary complex formed increases with an increase in the number of base pairs in the antisense dimerization domain, as shown in TABLE 3.

TABLE 3

Oligos (SEQ ID NOS:)[a]	Complex[b]	Tm, °C
10+14	CTAGAAGGAGAGAGATGGGTGCGAGAG CTTCCTCTCTCT CCCACGCTC G C G C C G	45.9
11+15	CTAGAAGGAGAGAGATGGGTGCGAGAG CTTCCTCTCTCT CCCACGCTC G C G C C G C G	47.3
12+16	CTAGAAGGAGAGAGATGGGTGCGAGAG CTTCCTCTCTCT CCCACGCTC G C G C C G C G G C	48.4
13+1	CTAGAAGGAGAGAGATGGGTGCGAGAG CTTCCTCTCTCT CCCACGCTC G C G C C G C G G C C G G C	53.2
9+14	CTAGAAGGAGAGAGATGGGTGCGAGAG *TTCCTCTCTCT*ACCCACGCTC *GC* *GC* *CG*	47.9

[a] 3' cooperative oligonucleotide then 5' cooperative oligonucleotide.

[b] Target (SEQ ID NO:21) is bolded and is 5' → 3'; complementary cooperative oligonucleotides are 3' → 5'

For example, the double helical complexes with 3 base pair (oligonucleotides 10+14), 4 base pair (oligonucleotides 11+15), 5 base pair (oligonucleotides 12+16), and 7 base pair (oligonucleotides 13+17) antisense dimerization domains gave Tms of 45.9°C, 47.3°C, 48.4°C and 53.2°C, respectively. Further increases in duplex stem length results in the formation of a stable complex between the two tandem oligonucleotides in the absence of the target sequence, an occurrence which is not desirable. In all the cases, a sharp cooperative single melting transition was observed (FIG. 3).

Modified cooperative oligonucleotides were studied for their antisense abilities. For example, phosphorothioate internucleotide-linked forms of cooperative oligonucleotides were studied for their ability to activate RNase H. RNase H is an enzyme that recognizes RNA-DNA heteroduplexes and hydrolyses the RNA component of the heteroduplex (Cedergren et al. (1987) *Biochem. Cell Biol.* 65:677). Some studies have shown that antisense oligonucleotides have less transition inhibition activity in RNase H-free systems than in systems where RNase H is present (Haeuptle et al. (1986) *Nucleic Acids Res.* 14:1427-14448; Minshull et al. (1986) *Nucleic Acids Res.* 14:6433-6451), or when the chemical modification on antisense oligonucleotide is unable to evoke RNase H activity (Maher III et al. (1988) *Nucl. Acids Res.* 16:3341-3358; Leonetti et al. (1988) *Gene* 72:323-332). In addition, it has also been

showed that a 4 to 6 base pair long hybrid is sufficient to evoke RNase H activity.

A 39mer RNA target sequence (SEQ ID NO:22) which encodes a portion of the HIV-1 *gag* gene (TABLE 1) was synthesized to study the RNase H activation property of modified cooperative oligonucleotides of the invention. As per the design, modified oligonucleotides 1, 10, and 17 bind to a 9 base site on the 3'-side of the binding site of the target, and modified oligonucleotides 2, 13, and 14 bind on the 5'-side of the target adjacent to the binding site of the former oligonucleotide. Oligonucleotide 5 binds to the entire length of the 21 bases on the target. Oligonucleotides 6, 7, 18 and 19 contained mismatches.

An autoradiogram showing the RNase H hydrolysis pattern of the RNA target in the absence and presence of oligonucleotides of the invention is shown in FIGS. 4A and 4B. As expected, in experiments 2 and 5 (FIG. 4A), and in experiment 2 (FIG. 4B), hydrolytic activity is observed towards the 3'-end of the target RNA (lower half of the autoradiogram) in which oligonucleotides 1, 14, and 17, respectively, are present. Similarly, in experiments 3 and 6 (FIG. 4A) and in experiment 3 (FIG. 4B), RNA degradation bands are present only in the upper half of the autoradiogram, indicating the binding of oligonucleotides 2, 10, and 13, respectively, on the 5'-side of the target. When combinations of oligonucleotides are present (i.e., 1+2, 10+14, and 13+17) in experiments 4 and 7 (FIG. 4A) and in experiment 4 (FIG. 4B), the RNase H

degradation pattern obtained is very similar to the one observed in the case of control oligonucleotide 5 in experiment 1 (FIGS. 5A and 5B). This clearly indicates that the new short tandem cooperative oligonucleotides of the invention bind to the target RNA as expected with sequence specificity and evoke RNase H activity.

To further understand sequence specificity of the cooperative oligonucleotides versus longer oligonucleotides, two short oligonucleotides analogous to oligonucleotide 1 having one and two mismatches, oligonucleotides 18 (SEQ ID NO:18) and 19 (SEQ ID NO:19), were synthesized and studied for RNase H activation in comparison to oligonucleotides 23 and 24. FIG. 5 shows the RNase H hydrolytic pattern of target RNA in the presence of the mismatched oligonucleotides. Oligonucleotide 23 (SEQ ID NO:23) with 1 mismatch (experiment 2) shows the same RNase H degradation pattern as completely matched oligonucleotide 5 (experiment 1). Oligonucleotide 24 (SEQ ID NO:24) with two mismatches (experiment 3) shows little or no RNA hydrolysis in the middle of the binding site, where the mismatches are located. However, on either side of the mismatches the degradation pattern is exactly like that found with oligonucleotide 5 which has no mismatches. This clearly indicates that, in spite of the two mismatches, oligonucleotide 24 binds to the target strongly 14 enough to activate RNase H. Oligonucleotide 18 with one mismatch (experiment 5) shows little or no RNA degradation compared to oligonucleotide 1 (experiment 4). However, it appears that oligonucleotide 18 has a strong binding

site on the 5'-end of the RNA target as indicated by the RNA degradation bands towards the 5'-end of the RNA. No digestion of the 3'-end of the RNA target and little digestion of the 5'-end was observed with oligonucleotide 19, which has two mismatches (experiment 6). This clearly demonstrates that the new cooperative oligonucleotides bind with sequence specifically.

Representative modified cooperative oligonucleotides of the invention were also studied for their HIV-1 virus inhibition properties in cell cultures. The results using phosphorothioate cooperative oligonucleotides are shown in FIG. 6 as a graph of percent virus inhibition versus concentration of the oligonucleotide(s) and FIG. 7. Oligonucleotide 5, a 21mer that is 4 bases shorter than oligonucleotide 20, demonstrated little or no significant activity up to a 15 μM concentration. Similarly, the combination of oligonucleotides 1+2, which bind to the same sequence on the target as oligonucleotide 5, also failed to show much activity. The IC_{50} for oligonucleotide 20 in the same assay system was about 0.55 μM. In contrast, a pronounced synergistic effect is observed with oligonucleotide combination 13+17 which forms a 7 base pair dimerization duplex stem. This oligonucleotide combination showed activity close to oligonucleotide 20, with an IC_{50} value of about 4.0 μM. The combination 10+4, which forms a three base pair extended dimerization stem, showed about 15% virus inhibition at 4 μM concentration (FIG. 7). Combination 12+16, with a five base extended dimerization domain, showed about 25% viral

inhibition at the same concentration (FIG. 7). Thus, the inhibition of HIV-1 virus progression by combinations of oligonucleotides is higher than the average of either oligonucleotide of the pair tested alone. Note that the concentration of each oligonucleotide in a combination is half that of the individual oligonucleotide tested alone. For example, the concentration of oligonucleotides 13 and 17 is
2 plus 2, to a total concentration of 4 μM, whereas the concentration of oligonucleotide 17, when it was tested alone, was 4 μM. The other oligonucleotides studied individually or in combinations did not show significant activity even up to 10 μM concentration (FIG. 7). The oligonucleotides 9+14, which form a 3 base pair duplex stem without a base separation between the binding oligonucleotides on the target, showed comparable activity to that of the combination of oligonucleotides 12 and 16, which form a 5 base pair duplex stem but with a one base separation. This result correlates well with the Tm data (Table 3).

The oligonucleotide combinations with an extended dimerization domain inhibited HIV much more efficiently than oligonucleotide 5 or the combination of oligonucleotides 1 and 2. FIG. 8 shows the relationship between HIV-1 inhibition and Tm of the complex formed. The oligonucleotide combination 13 and 17, which forms a 7 base pair antisense duplex stem, showed significantly greater activity relative to the other combinations of oligonucleotides, which form 3, 4, and 5 base pair duplex stems and oligonucleotide 5, a 21-mer.

		CGTCCCTTTGGTAAACATACCT	51
	39	TCCATTCAAATGGTTTGCCTGC	
		AGGTAAGTTTACCAAAAGGACG	44, 52
		CC	
		CG	
		TA	
		CG	
		CC	
	39	TCCATTCAAATGGTTTGCCTGC	
		AGGTATGTTTACCAAACGGACG	53, 54
		CC	
		CG	
		TA	
		CG	
		CC	
	39	TCCATTCAAATGGTTTGCCTGC	
		AGGTATGTTTACCAAAAGGACG	53, 52
		CC	
		CG	
		TA	
		CG	
		CC	
	38	GCAGGCAAACCATTTGAATGGA	
		CGTCCGTTTGGTAAACATACCT	46, 55
		CC	
		TA	
		CG	
		CC	
		CG	
	38	GCAGGCAAACCATTTGAATGGA	
		CGTCCCTTTGGTAAACTTACCT	56, 47
		CC	
		TA	
		CG	
		CC	
		CG	
	38	GCAGGCAAACCATTTGAATGGA	
		CGTCCCTTTGGTAAACATACCT	56, 55
		CC	
		TA	
		CG	
		CC	
		CG	

* = underlined bases represent mismatches

' = Target is bolded and is 5' → 3'; cooperative oligonucleotides are 3' → 5'.

In addition, sequence-specific and cooperative binding of short oligonucleotides that bind to adjacent sites are useful to target sequences with point mutations specifically. In addition, undesirable non-sequence specific effects can be

These results demonstrate that modified cooperative oligonucleotides with dimerization domains have an enhanced ability to inhibit the expression of the target gene.

As described above, cooperative oligonucleotides of the invention can be directed to any target oligonucleotide. Other non-limiting examples of such cooperative oligonucleotides include those specific for influenza nucleic acid targets which are shown below in Table 4.

TABLE 4

Target Oligo (SEQ ID NO:)	Complex[a]	Cooperative Oligo (SEQ ID NO:)
38	GCAGGCAAACCATTTGAATGGA CGTCCGTTTGGTAAACTTACCT	41, 48
39	TCCATTCAAATGGTTTGCCTGC AGGTAAGTTTACCAAACGGACG	42, 43
39	TCCATTCAAATGGTTTGCCTGC AGGTAAGTTTACCAAACGGACG CC CG TA CG CC	44, 45
38	GCAGGCAAACCATTTGAATGGA CGTCCGTTTGGTAAACTTACCT CC TA CG CC CG	46, 47
39	TCCATTCAAATGGTTTGCCTGC AGGTAAGTTTACCAAAAGGACG	48

TABLE 4 (con't)

Target Oligo (SEQ ID NO:)	Complex[a]	Cooperative Oligo (SEQ ID NO:)
39	TCCATTCAAATGGTTTGCCTGC AGGTATGTTTACCAAAAGGACG	49
38	GCAGGCAAACCATTTGAATGGA CGTCCGTTTGGTAAACATACCT	50
38	GCAGGCAAACCATTTGAATGGA	

reduced by using at least two short oligonucleotides that can bind to a longer target sequence rather than one long oligonucleotide that binds to the same length of the target sequence. For example, long oligonucleotides that contain a modified backbone, such as phosphorothioates, activate complement, which have adverse cardiovascular effects (Galbraith et al. (1994) *Antisense Res. Dev.* 4:201-207; and Cornish et al. (1993) *Pharmacol. Commun.* 3:239-247). In conclusion, combination oligonucleotides represent an alternative therapeutic strategy to the use of a single oligonucleotide, in cases in which use of the latter is limited by concentration and chain length constraints, and the associated problems of toxicity and production costs.

The synthetic cooperative oligonucleotides of the invention also may be used to identify the presence of the nucleic acids of a particular virion or bacteria in cell cultures, for example, by labelling the oligonucleotide and screening for double-stranded, labelled DNA in the cells by *in situ* hybridization or some other art-recognized detection method.

In addition, the function of various genes in an animal, including those essential to animal development can be examined using the cooperative oligonucleotides of the invention. Presently, gene function can only be examined by the arduous task of making a "knock out" animal such as a mouse. This task is difficult, time-consuming and cannot be accomplished for genes essential to animal development since the "knock out" would produce a

lethal phenotype. The present invention overcomes the shortcomings of this model.

It is known that antisense oligonucleotides can bind to a target single-stranded nucleic acid molecule according to the Watson-Crick or the Hoogsteen rule of base pairing, and in doing so, disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal transcription, splicing, or translation; by triggering the enzymatic destruction of mRNA by RNase H if a contiguous region of deoxyribonucleotides exists in the oligonucleotide, and/or by destroying the target via reactive groups attached directly to the antisense oligonucleotide.

Thus, because of the properties described above, such oligonucleotides are useful therapeutically by their ability to control or down-regulate the expression of a particular gene in a cell, e.g., in a cell culture or in an animal, according to the method of the present invention.

The cooperative oligonucleotides of the invention may also be used to inhibit transcription of any gene in a cell, including a foreign gene. For example, the cooperative oligonucleotides as provided by the invention may be use to inhibit the expression of HIV genes within infected host cells and thus to inhibit production of HIV virions by those cells. The synthetic oligonucleotides of the invention are thus useful for treatment of HIV infection and AIDS in mammals, particularly the

treatment of mammals used as animal models to study HIV infection and AIDS. The synthetic oligonucleotides of the invention are also useful for treatment of humans infected with HIV and those suffering from AIDS.

As discussed above, the synthetic oligonucleotides of the invention may be used as a pharmaceutical composition when combined with a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration. Such a composition may contain, in addition to the synthetic oligonucleotide and carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The pharmaceutical composition of the invention may also contain other active factors and/or agents which enhance inhibition of virus or bacterial production by infected cells. For example, combinations of synthetic oligonucleotides, each of which inhibits transcription of a different HIV gene, may be used in the pharmaceutical compositions of the invention. The pharmaceutical composition of the invention may further contain nucleotide analogs such as azidothymidine, dideoxycytidine, dideotyinosine, and the like. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with the synthetic oligonucleotide of the invention, or to minimize side-effects caused by the synthetic oligonucleotide

of the invention. Conversely, the synthetic oligonucleotide of the invention may be included in formulations of a particular anti-HIV factor and/or agent to minimize side effects of the anti-HIV factor and/or agent.

The pharmaceutical composition of the invention may be in the form of a liposome in which the synthetic oligonucleotides of the invention is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which are in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; and U.S. Patent No. 4,737,323.

The pharmaceutical composition of the invention may further include compounds which enhance delivery of oligonucleotides into cells, as described in WO 95/32739.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, e.g., healing of chronic conditions

characterized by HIV and associated infections and complications or by other viral infections or increase in rate of healing of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of one or more of the synthetic oligonucleotide of the invention is administered to a mammal infected with HIV. The synthetic oligonucleotide of the invention may be administered in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing cytokines, lymphokines, other hematopoietic factors, other anti-viral agents, and the like. When co-administered with one or more cytokines, lymphokines or other hematopoietic factors, other anti-viral agents, the synthetic oligonucleotide of the invention may be administered either simultaneously with the cytokine(s), lymphokine(s), other hematopoietic factor(s), other antiviral agents, and the like, or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering the synthetic oligonucleotide of the invention in combination with cytokine(s), lymphokine(s), other hematopoietic factor(s), anti-viral agents, and the like.

Administration of the synthetic oligonucleotide of the invention used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, or cutaneous, subcutaneous, or intravenous injection. Intravenous administration to the patient is preferred.

When a therapeutically effective amount of synthetic oligonucleotide of the invention is administered orally, the synthetic oligonucleotide will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% synthetic oligonucleotide and preferably from about 25 to 90% synthetic oligonucleotide. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of the synthetic oligonucleotide and preferably from about 1 to 50% synthetic oligonucleotide.

When a therapeutically effective amount of synthetic oligonucleotide of the invention is administered by intravenous, cutaneous or subcutaneous injection, the synthetic oligonucleotide will preferably be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to the synthetic oligonucleotide, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

The amount of synthetic oligonucleotide in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of synthetic oligonucleotide with which to treat each individual patient. Initially, the attending physician will administer low doses of the synthetic oligonucleotide and observe the patient's response. Larger doses of synthetic oligonucleotide may be administered until the optimal therapeutic

effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 1 ng to about 100 mg of synthetic oligonucleotide per kg body weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the synthetic oligonucleotide will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately, the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

EXAMPLES

1. Cooperative Oligonucleotide Synthesis

Cooperative oligodeoxyribonucleotides were synthesized on a Milligen 8700 DNA synthesizer using β-cyanoethylphosphoramidite chemistry (*Meth. Mol. Biol.*

(1993) Vol. 20 (Agrawal (ed.) Humana Press, Totowa, NJ, pp. 33-61) on a (500 Å controlled pore glass solid support). Monomer synthons and other DNA synthesis reagents were obtained from Milligen Biosearch (Burlington, MA). After the synthesis and deprotection, oligonucleotides were purified on reverse phase (C_{18}) HPLC, detritylated, desalted (Waters C_{18} sep-pack cartridges (Waters, Milford, MA), and checked for purity by polyacrylamide gel electrophoresis (Manniatis et al. in *Molecular Cloning (A Laboratory Manual)*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Cooperative oligoribonucleotides and hybrids (RNA/DNA) cooperative oligonucleotides are prepared according to the method(s) of Metelev et al. (*FEBS. Lett.* (1988) 226:232-234; and Atabekov et al. (1988) *FEBS. Lett.* 232:96-98.

Cooperative phosphorothioate oligonucleotides for RNase H and tissue culture experiments were synthesized as above but using sulfurizing agent as oxidant instead of normal iodine oxidant. Post-synthetic processing was carried out exactly as above but desalting was performed by dialysis for 72 hours against double distilled water.

Other modified forms of the cooperative oligonucleotides are prepared as described in Agrawal (ed.) (*Meth. Mol. Biol.*, Vol. 20, *Protocols for Oligonucleotides and Analogs*, (1993) Humana Press, Totowa, NJ).

2. UV Melting Studies

UV melting experiments were carried out in 150 mM sodium chloride, 10 mM sodium dihydrogen phosphate, and 2 mM magnesium chloride, pH 7.4 buffer. The oligonucleotide concentration was 0.36 μM as single strand. The oligonucleotides were mixed in buffer, heated to 95°C, cooled down to room temperature, and left at 4°C overnight. Thermal denaturation profiles were recorded at 260 nm at a heating rate of 0.5°C/min on a spectrophotometer (Perkin-Elmer Lamba2, (Norwalk CT) equipped with a peltier thermal controller and attached to a personal computer for data collection. The (Tm) melting temperatures were measured from first derivative plots (dA/dT vs T). Each value is an average of two separate runs and the values are within ±1.0°C range.

3. RNase H Assay

An RNA target (SEQ ID NO:22) was labelled at its 3'-end using terminal transferase and [α-^{32}P]ddATP (Amersham, (Arlington Heights, IL) using standard protocols (Manniatis et al. in *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). End-labelled RNA (3000-5000 cpm) was incubated with 1 to 1.5 ratio of the oligonucleotides in 30 μl of 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM KCl, 0.1 mM DTT, 5% sucrose (w/v), and 40 units of RNasin (Promega, Madison, WI) at 4°C overnight. An aliquot (7 μl) was taken out as control, 1 μl (0.8 unit) of *E. coli* RNase H (Promega, Madison, WI) was added to the remaining reaction mixture and incubated at room temperature. Aliquots (7 μl) were taken out at

different time intervals. The samples were then analyzed on a 7 M urea 20% polyacrylamide gel. After the electrophoresis, an autoradiogram was developed by exposing the gel to Kodak X-Omat AR film at -70°C.

4. Antiviral Assay

The effect of the antisense oligonucleotides on the replication of HIV-1 during an acute infection was determined. The test system is a modification of the standard cytopathic effect (CPE)-based MT-2 cell assay (Posner et al. (1991) *J. Immunol.* 146:4325; Pawels et al. (1988) *J. Virol. Methods* 20:309; Mosmann (1983) *J. Immunol. Methods* 65:55). Briefly, serial dilutions of antisense oligonucleotides synthesized as described above, or the combinations of such oligonucleotides, were prepared in 50 μM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin), in triplicate, in 96-well plates. Virus, (HIV-1 IIIB originally obtained from Dr. Robert Gallo, NCI (Popovic et al. (1984) *Science* 224:497) and propagated in H9 cells (Gazdar et al. (1980) *Blood* 55:409) by the method of Vujcic (*J. Infect. Dis.* (1988) 157:1047), diluted to contain a 90% cytopathic effect (CPE) dose of virus in 50 μl, was added followed by 100 μl of 4 x 10^5/ml MT-2 cells (Harada et al. (1985) *Science* 229:563) in complete medium. The plates were incubated at 37°C in 5% CO_2, for 5 days. 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; thiazoyl blue (MTT) dye (Sigma, St. Louis, MO) was added and quantitated at OD_{540}-OD_{690} as described (Posner et al. (1991) *J.*

Immunol. 146:4325). Percent viral inhibition was calculated by the formula: (experimental-virus control)/(medium control-virus control) x 100.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kandimalla, Ekambar R.
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(ii) TITLE OF INVENTION: COOPERATIVE OLIGONUCLEOTIDES

(iii) NUMBER OF SEQUENCES: 57

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCGCACCC 9

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATCTCTCTCC TT 12

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCTCTCTCCT TC 12

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTCTCTCCTT CT 12

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCGCACCCA TCTCTCTCCT T 21

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCGCACCCG TCTCTCTCCT TC 22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTCTCAACCA TCTCTCTCCT T 21

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGAGGCUAGA AGGAGAGAGA UGGGUGCGAG AGCGU 35

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GACCACTTCT CCTTCT 16

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GAATGAGCCA TCTCAGTGGT C 21

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTCTCGCACC TCACTC 16

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCACTTCTCC TTCT 14

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATGAGCCATC TCAGTGG 17

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTCTCGCACC TCAC 14

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ACTTCTCCTT CT 12

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GAGCCATCTC AGT 13

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CTCTCGCACC TC 12

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TCTCCTTCT 9

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCATCTC 7

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CTCTCGCAC 9

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GCAGGCAAAC CATTTGAATG GA 22

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

TCCATTCAAA TGGTTTGCCT GC 22

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TCCATTCAAA T 11

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GGTTTGCCTG C 11

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ATTTGAATGG A 11

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GCAGGCAAAC C 11

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GCTCGATTTG AATGGA 16

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GCAGGCAAAC CCGAGC 16

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CGCTGGGTTT GCCTGC 16

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TCCATTCAAA TCAGCG 16

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GCAGGAAAAC CATTTGAATG GA 22

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GCAGGAAAAC CATTTGTATG GA 22

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TCCATACAAA TGGTTTGCCT GC 22

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TCCATACAAA TGGTTTCCCT GC 22

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

GCAGGAAAAC CCGAGC 16

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

GCTCGATTTG TATGGA 16

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

GCAGGCAAAC CCGAGC 16

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

TCCATACAAA TCAGCG 16

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CGCTGGGTTT CCCTGC 16

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

TCTCTCTCCT TC 12

What is claimed is:

1. A composition comprising at least a first synthetic cooperative oligonucleotide and a second synthetic cooperative oligonucleotide, each oligonucleotide comprising a region complementary to one of tandem, non-overlapping regions of a target nucleic acid, and a dimerization domain at a terminus of each of the oligonucleotides,

the dimerization domains of the oligonucleotides being complementary to each other, and

the target nucleic acid being an mRNA, a single-stranded viral DNA, or a single-stranded viral RNA.

2. The composition of claim 1 wherein the oligonucleotides are complementary to tandem regions of the target nucleic acid that are separated by 0 to 3 bases.

3. The composition of claim 1 wherein each of the oligonucleotides are about 9 to 25 nucleotides in length.

4. The composition of claim 1 wherein the dimerization domain of the first cooperative oligonucleotide is located at its 3' terminal portion, and is complementary to the dimerization domain of the second oligonucleotide which is located at its 5' terminal portion.

5. The composition of claim 1 wherein the dimerization domain of a first cooperative oligonucleotide is located at its 3' terminal portion, and is complementary to the dimerization domain of a second oligonucleotide which is located at its 3' terminal portion.

6. The composition of claim 1 wherein the dimerization domain of a first cooperative oligonucleotide is located at its 5' terminal portion, and is complementary to the dimerization domain of a second oligonucleotide which is located at its 5' terminal portion.

7. The composition of claim 1 wherein the dimerization domain of each of the oligonucleotides are 3 to 7 nucleotides in length.

stem

8. The composition of claim 1 wherein the first and second regions of the nucleic acid that are separated by 0 to 3 bases.

9. The composition of claim 1 wherein at least one of the oligonucleotides is modified.

10. The composition of claim 9 wherein at least one of the oligonucleotides contains at least one non-phosphodiester internucleotide linkage.

11. The composition of claim 9 wherein at least one of the oligonucleotides contains at least one phosphorothioate internucleotide linkage.

12. The composition of claim 1 wherein first and second oligonucleotides are hybridized to the target nucleic acid and the dimerization domain of the first oligonucleotide is hybridized to the dimerization domain of the second oligonucleotide.

13. A duplex structure comprising a first synthetic cooperative oligonucleotide and a second synthetic cooperative oligonucleotide, each oligonucleotide comprising a region complementary to one of tandem, non-overlapping regions of a target nucleic acid,

the target nucleic acid being an mRNA, a single-stranded viral RNA, or a single-stranded viral DNA, and

the first oligonucleotide having a terminal dimerization domain complementary and hybridized to a dimerization domain of the second oligonucleotide when the first and second oligonucleotides are hybridized to the target nucleic acid.

14. The structure of claim 13 wherein each of the oligonucleotides are about 9 to 25 nucleotides in length.

15. The structure of claim 13 wherein the dimerization domains of the first and second oligonucleotides each comprise about 3 to 7 nucleotides.

16. The structure of claim 13 wherein the first and second oligonucleotides are complementary to one of tandem regions of the target nucleic acid that are separated by 0 to 3 bases.

17. The structure of claim 13 hybridized to the target nucleic acid.

18. A composition comprising at least a first synthetic cooperative oligonucleotide, a second synthetic cooperative oligonucleotide, and a third synthetic cooperative oligonucleotide, each oligonucleotide comprising a region complementary to one of tandem, non-overlapping regions of a target nucleic acid, and a dimerization domain at one or both termini of each of the oligonucleotides,

the dimerization domain of the first and second oligonucleotide is at a single terminus of the first and second cooperative oligonucleotides, and a third cooperative oligonucleotide has a dimerization domain at both of its termini,

the dimerization domain of the first cooperative oligonucleotide being complementary to one dimerization domain of the third cooperative oligonucleotide, and the dimerization domain of the second cooperative oligonucleotide being complementary to the second dimerization domain of the third cooperative oligonucleotide, and

the target nucleic acid being an mRNA, a single-stranded viral DNA, or a single-stranded viral RNA.

19. The composition of claim 18 wherein the oligonucleotides are complementary to tandem regions

of the target nucleic acid that are separated by 0 to 3 bases.

20. The composition of claim 18 wherein each of the oligonucleotides are about 9 to 25 nucleotides in length.

21. The composition of claim 18 wherein the dimerization domain of each of the oligonucleotides are 3 to 7 nucleotides in length.

22. The composition of claim 18 wherein at least one of the oligonucleotides is modified.

23. The composition of claim 22 wherein at least one of the oligonucleotides contains at least one non-phosphodiester internucleotide linkage.

24. The composition of claim 23 wherein at least one of the oligonucleotides contains at least one phosphorothioate internucleotide linkage.

25. The composition of claim 18 wherein the dimerization domain of the first cooperative oligonucleotide is located at its 3' terminus and is complementary to the dimerization domain at the 5' terminus of the third oligonucleotide, and the dimerization domain of the second cooperative oligonucleotide is located at its 5' terminus and is complementary to the dimerization domain at the 3' terminus of the third oligonucleotide.

26. The composition of claim 18 wherein the dimerization domain of the first cooperative

oligonucleotide is located at its 5' terminus and is complementary and hybridized to the dimerization domain at the 3' terminus of the third oligonucleotide, and the dimerization domain of the second cooperative oligonucleotide is located at its 3' terminus and is complementary and hybridized to the dimerization domain at the 5' terminus of the third cooperative oligonucleotide.

27. A ternary structure comprising a first synthetic cooperative oligonucleotide, a second synthetic cooperative oligonucleotide, and a third synthetic cooperative oligonucleotide, each oligonucleotide comprising a region complementary to one of tandem, non-overlapping regions of a target nucleic acid, and each comprising a dimerization domain at one or both of their termini,

the dimerization domain of the first oligonucleotide being complementary and hybridized to a first dimerization domain at one terminus of the third oligonucleotide and the dimerization domain of the second oligonucleotide being complementary and hybridized to a second dimerization domain at the other terminus of the third oligonucleotide when the first, second, and third oligonucleotides are hybridized to the target nucleic acid, and

the target nucleic acid being an mRNA, a single-stranded viral RNA, or a single-stranded viral DNA.

28. The structure of claim 27 wherein each of the oligonucleotides are about 9 to 25 nucleotides in length.

29. The structure of claim 27 wherein the dimerization domains of the first and second oligonucleotides each comprise about 3 to 7 nucleotides.

30. The structure of claim 27 wherein the first and second oligonucleotides are complementary to one of tandem regions of the target nucleic acid that are separated by 0 to 3 bases.

31. The ternary structure of claim 27 hybridized to the target nucleic acid.

32. A pharmaceutical formulation comprising the composition of claim 1 and a physiologically acceptable carrier.

33. A pharmaceutical formulation comprising the duplex structure of claim 13 and a physiologically acceptable carrier.

34. A pharmaceutical formulation comprising the composition of claim 18 and a physiologically acceptable carrier.

35. A pharmaceutical formulation comprising the composition of claim 27 and a physiologically acceptable carrier.

36. A method of inhibiting the expression of a nucleic acid comprising the step of contacting the nucleic acid with the composition of claim 1.

37. A method of inhibiting the expression of a nucleic acid comprising the step of contacting the nucleic acid with the composition of claim 13.

38. A method of inhibiting the expression of a nucleic acid comprising the step of contacting the nucleic acid with the composition of claim 18.

39. A method of inhibiting the expression of a nucleic acid comprising the step of contacting the nucleic acid with the composition of claim 27.

40. The method of claim 36 wherein the nucleic acid is a viral nucleic acid.

41. The method of claim 40 wherein the nucleic acid is an HIV DNA or an HIV RNA.

42. The method of claim 40 wherein the nucleic acid is an influenza DNA or influenza RNA.

43. The method of claim 37 wherein the nucleic acid is a viral nucleic acid.

44. The method of claim 43 wherein the nucleic acid is an HIV DNA or an HIV RNA.

45. The method of claim 43 wherein the nucleic acid is an influenza DNA or influenza RNA.

46. The method of claim 38 wherein the nucleic acid is a viral nucleic acid.

47. The method of claim 46 wherein the nucleic acid is an HIV DNA or an HIV RNA.

48. The method of claim 46 wherein the nucleic acid is an influenza DNA or influenza RNA.

49. The method of claim 39 wherein the nucleic acid is a viral nucleic acid.

50. The method of claim 49 wherein the nucleic acid is an HIV DNA or an HIV RNA.

51. The method of claim 49 wherein the nucleic acid is a viral nucleic acid.

52. A method of treating viral infection comprising the step of administering to the infected subject the pharmaceutical composition of claim 32.

53. A method of treating viral infection comprising the step of administering to the infected subject the pharmaceutical composition of claim 33.

54. A method of treating viral infection comprising the step of administering to the infected subject the pharmaceutical composition of claim 34.

55. A method of treating viral infection comprising the step of administering to the infected subject the pharmaceutical composition of claim 35.

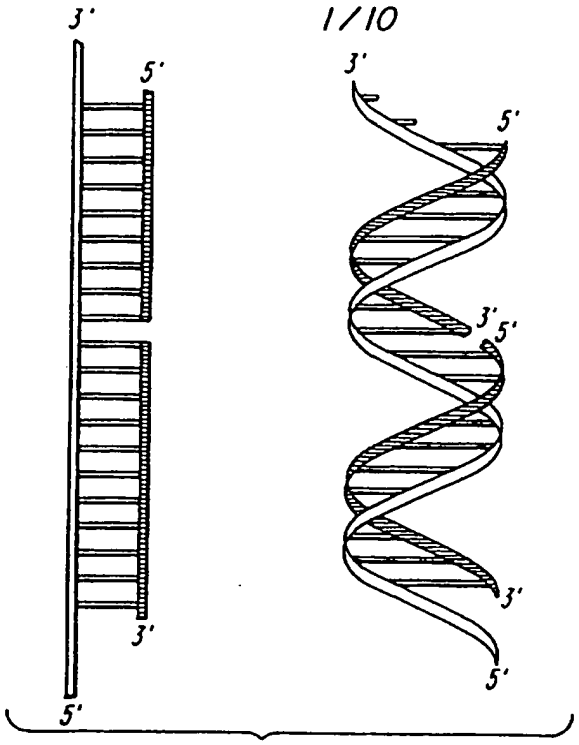

FIG. 1A

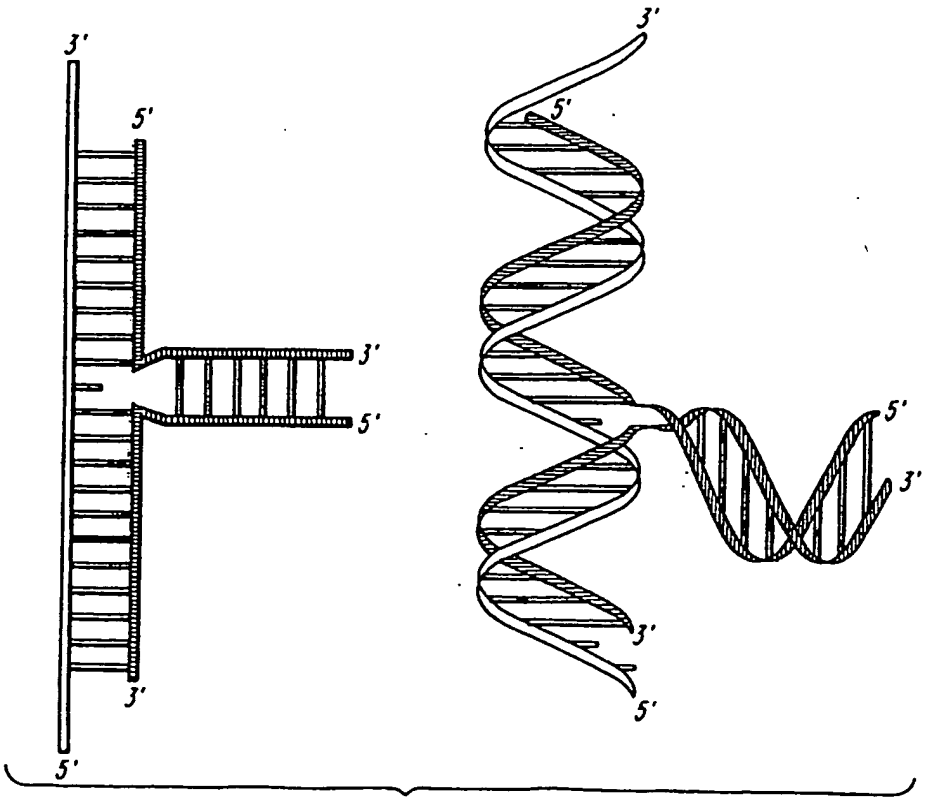

FIG. 1B

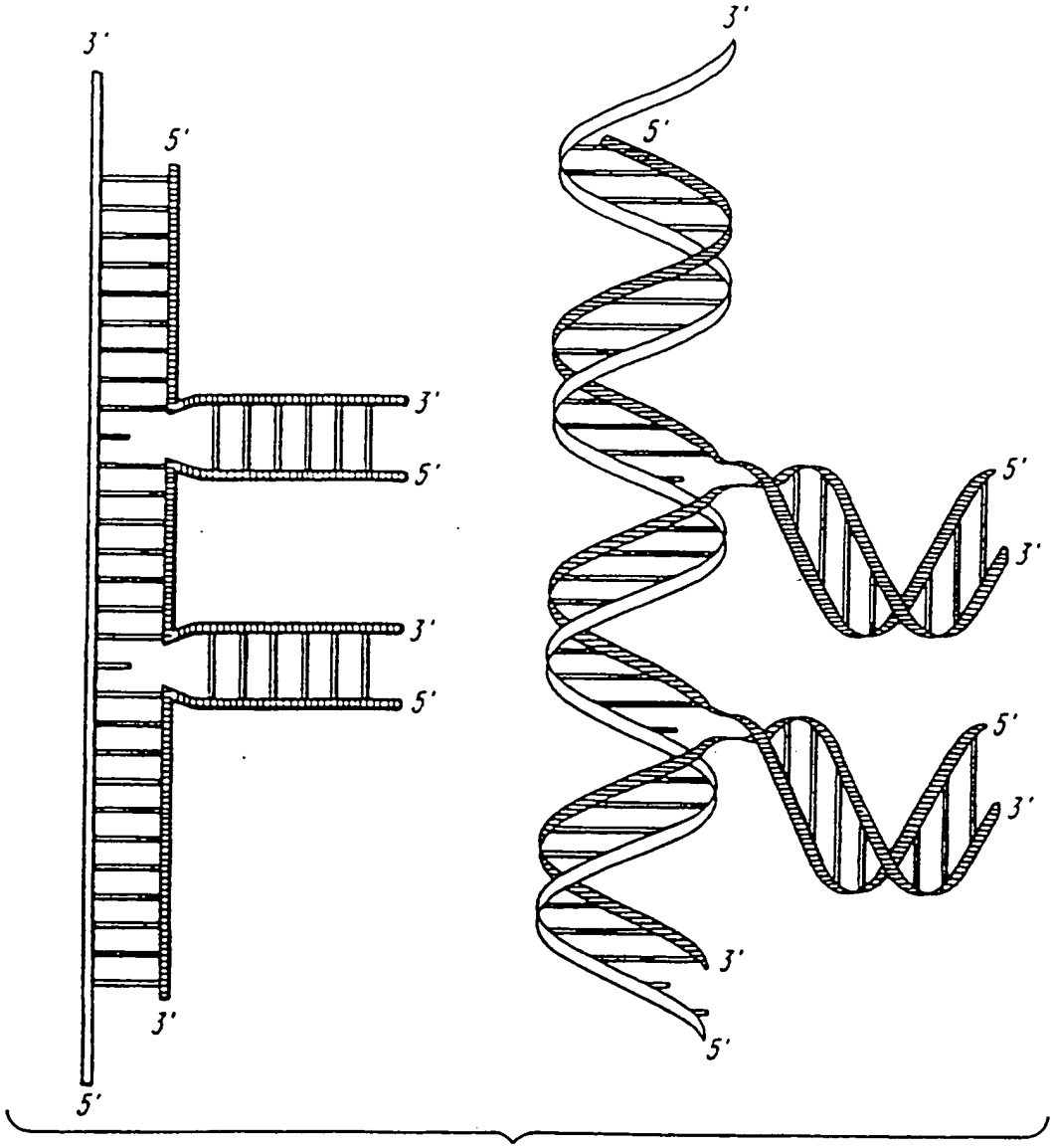

FIG. 1C

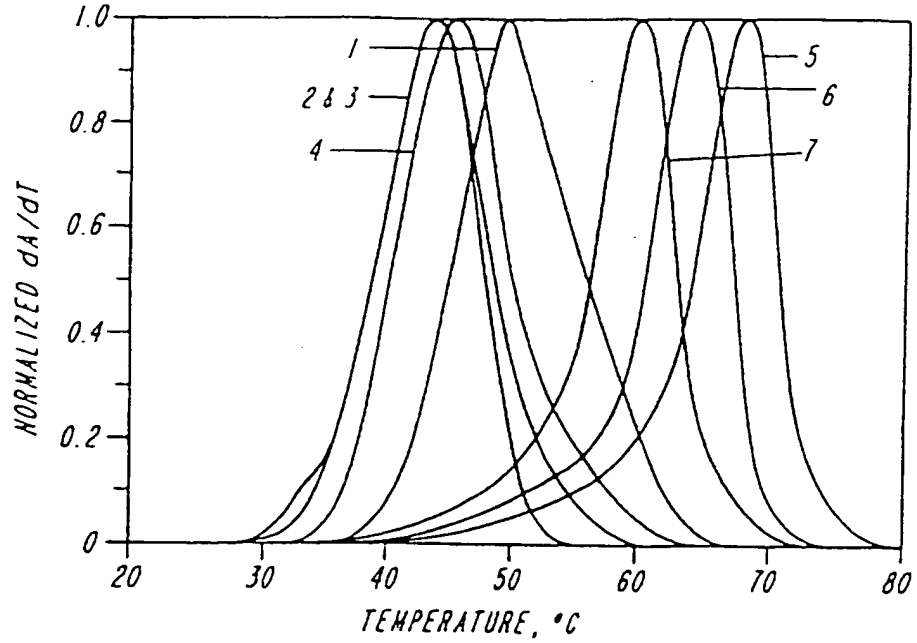
1.0
0.8
0.6
0.4
0.2
0
NORMALIZED dA/dT
1
2 & 3
4
5
6
7
20
30
40
50
60
70
80
TEMPERATURE, °C

FIG. 2A

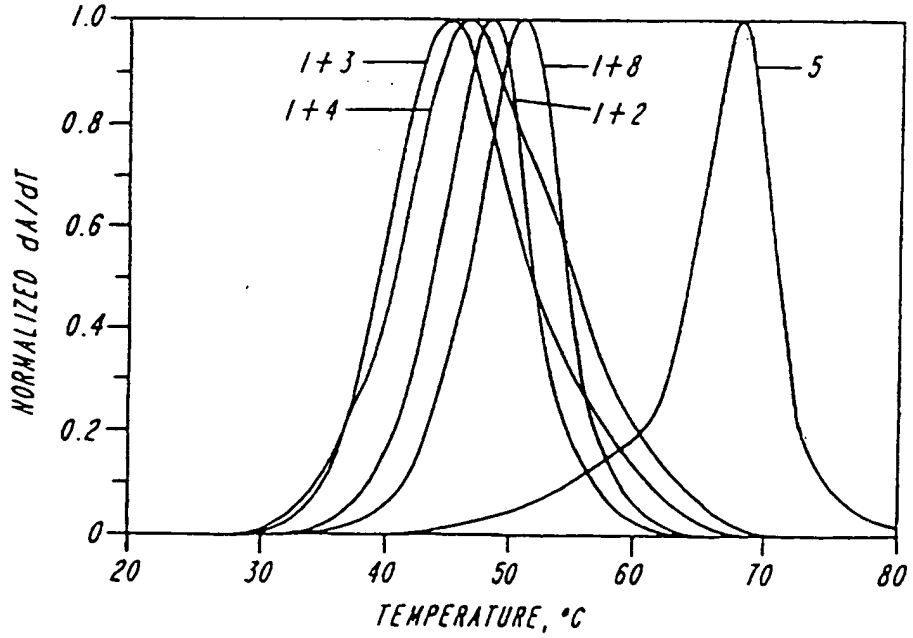
1.0
0.8
0.6
0.4
0.2
0
NORMALIZED dA/dT
1+3
1+4
1+8
1+2
5
20
30
40
50
60
70
80
TEMPERATURE, °C

FIG. 2B

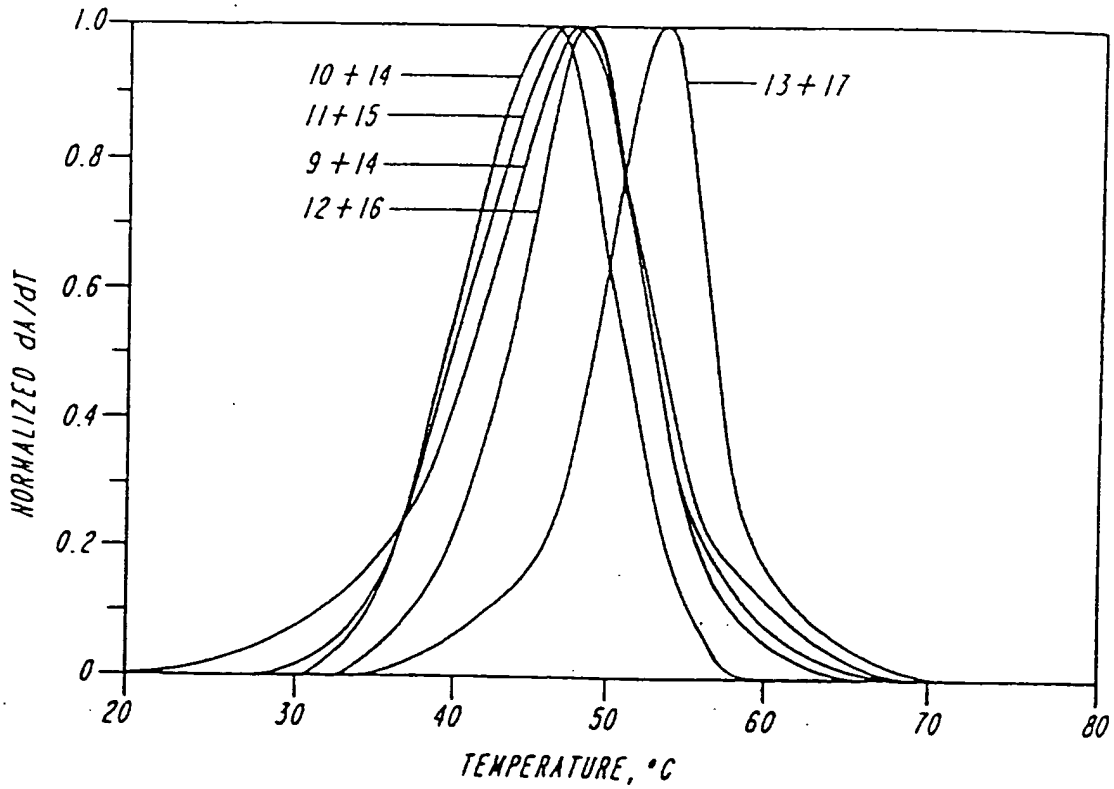
1.0
0.8
0.6
0.4
0.2
0
NORMALIZED dA/dT
10 + 14
11 + 15
9 + 14
12 + 16
13 + 17
20
30
40
50
60
70
80
TEMPERATURE, °C

FIG. 3

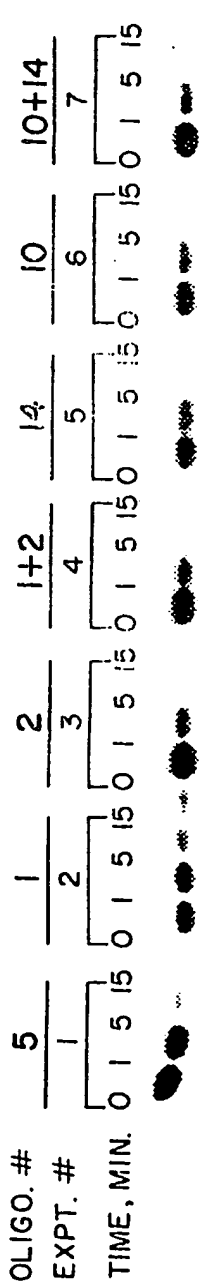
OLIGO. #
EXPT. #
TIME, MIN.
5
1
0 1 5 15
1
2
0 1 5 15
2
3
0 1 5 15
1+2
4
0 1 5 15
14
5
0 1 5 15
10
6
0 1 5 15
10+14
7
0 1 5 15

FIG. 4A

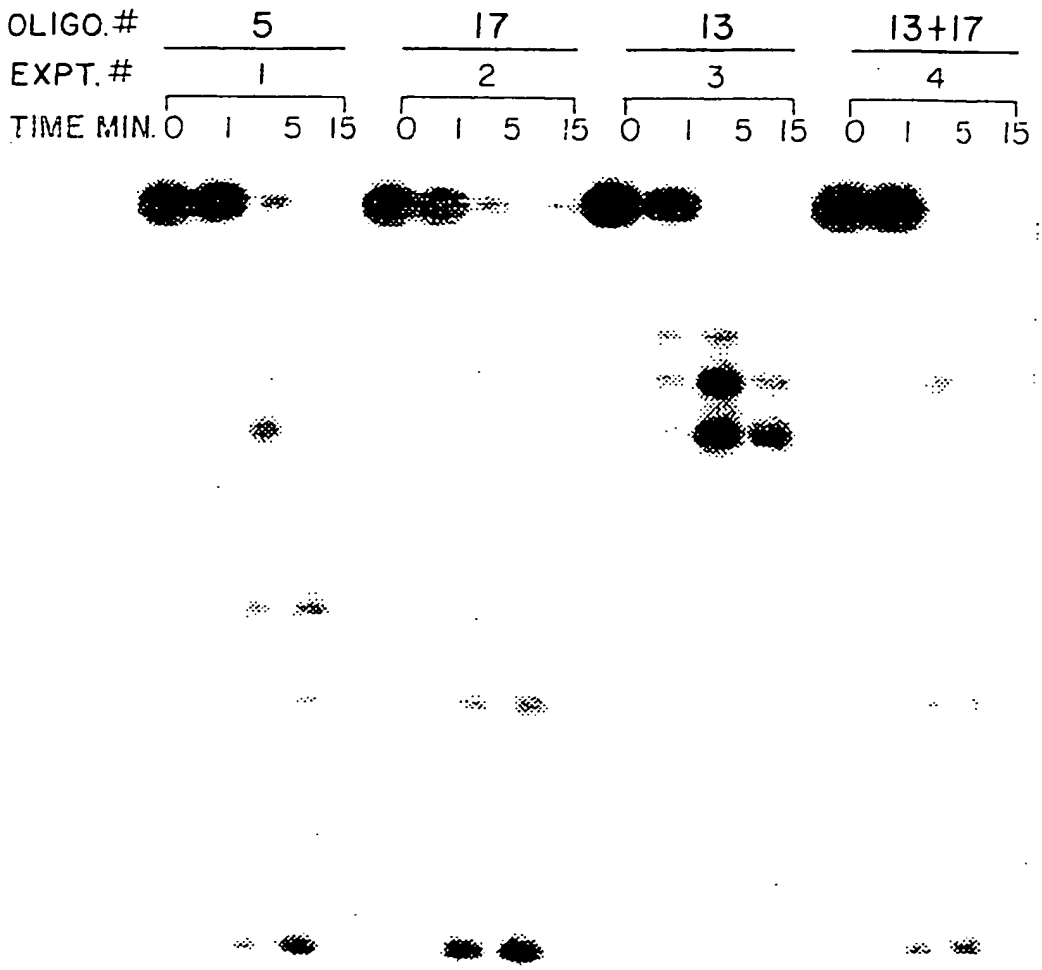

FIG. 4B

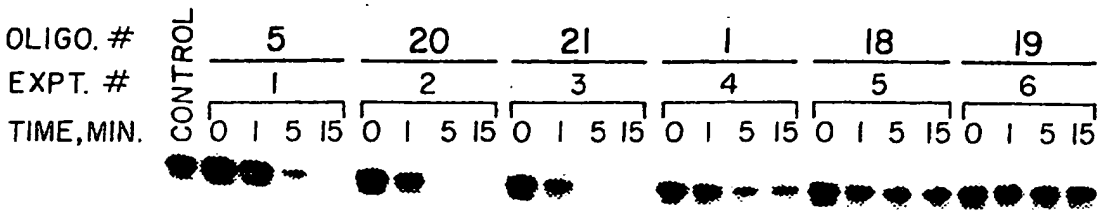
OLIGO. #
EXPT. #
TIME, MIN.
CONTROL
5
20
21
1
18
19
1
2
3
4
5
6
0 1 5 15 0 1 5 15 0 1 5 15 0 1 5 15 0 1 5 15 0 1 5 15

FIG. 5

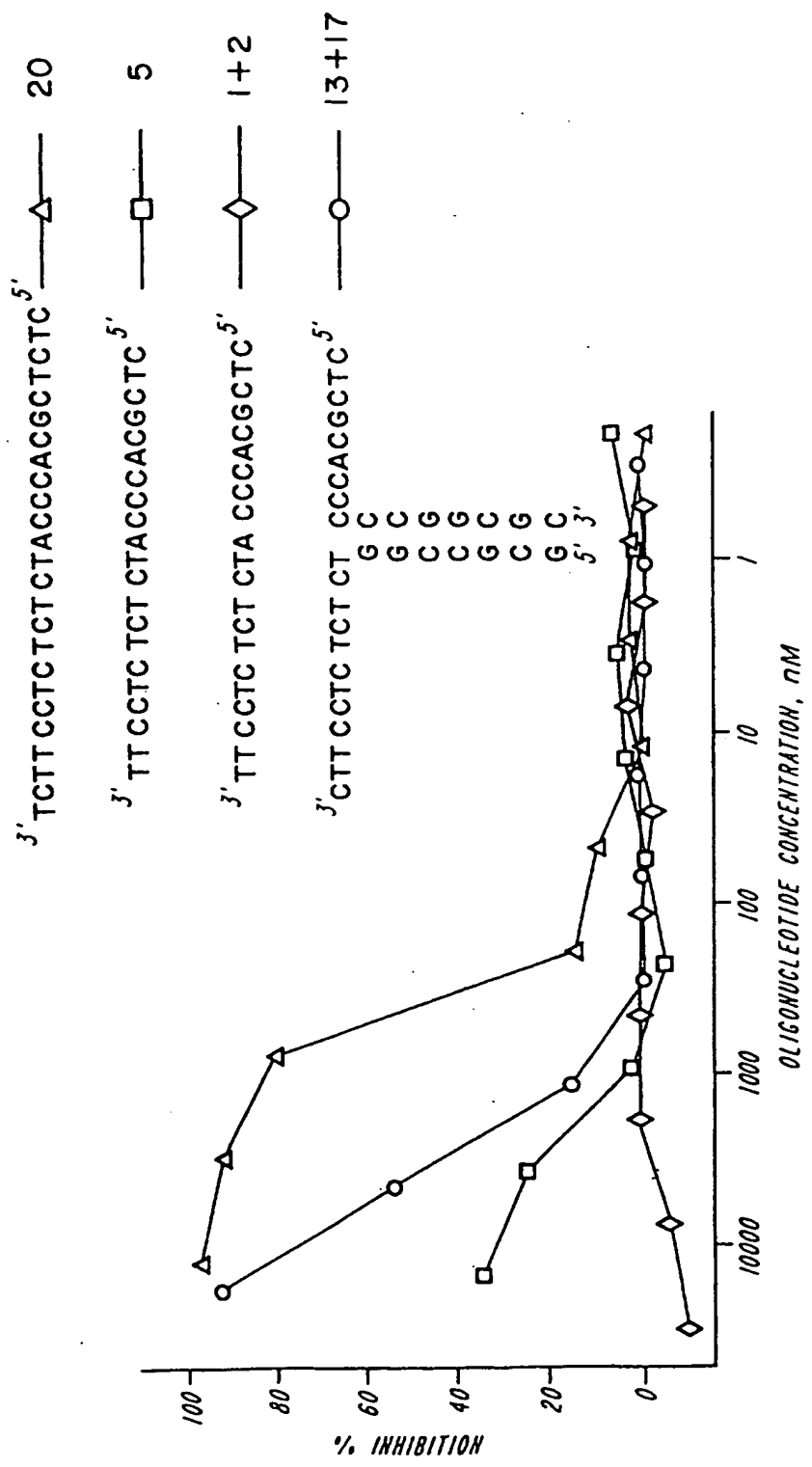
3' TCTT CCTC TCT CTA CCCACGCTCTC 5' 20
3' TT CCTC TCT CTA CCCACGCTC 5' 5
3' TT CCTC TCT CTA CCCACGCTC 5' 1+2
3' CTT CCTC TCT CT CCCACGCTC 5' 13+17
G C
G C
C G
C G
G C
C G
G C
5' 3'
% INHIBITION
100
80
60
40
20
0
10000
1000
100
10
1
OLIGONUCLEOTIDE CONCENTRATION, nM

FIG. 6

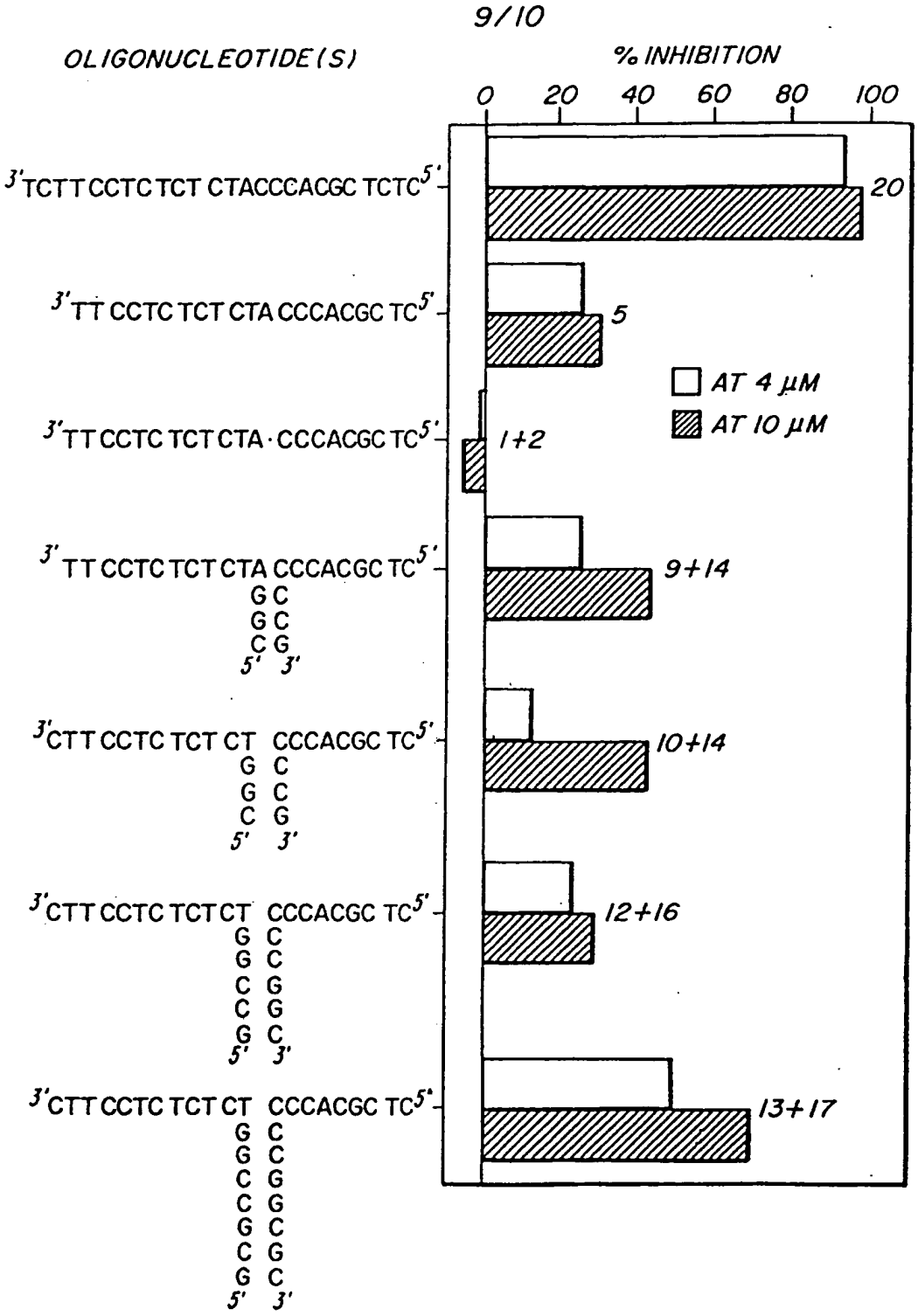
OLIGONUCLEOTIDE(S)
% INHIBITION
0 20 40 60 80 100
3'TCTT CCTC TCT CTACCCACGC TCTC5'
20
3'TT CCTC TCT CTA CCCACGC TC5'
5
AT 4 μM
AT 10 μM
3'TT CCTC TCT CTA·CCCACGC TC5'
1+2
3' TT CCTC TCT CTA CCCACGC TC5'
G C
G C
C G
5' 3'
9+14
3'CTT CCTC TCT CT CCCACGC TC5'
G C
G C
C G
5' 3'
10+14
3'CTT CCTC TCT CT CCCACGC TC5'
G C
G C
C G
C G
G C
5' 3'
12+16
3'CTT CCTC TCT CT CCCACGC TC5'
G C
G C
C G
C G
G C
C G
G C
5' 3'
13+17

FIG. 7

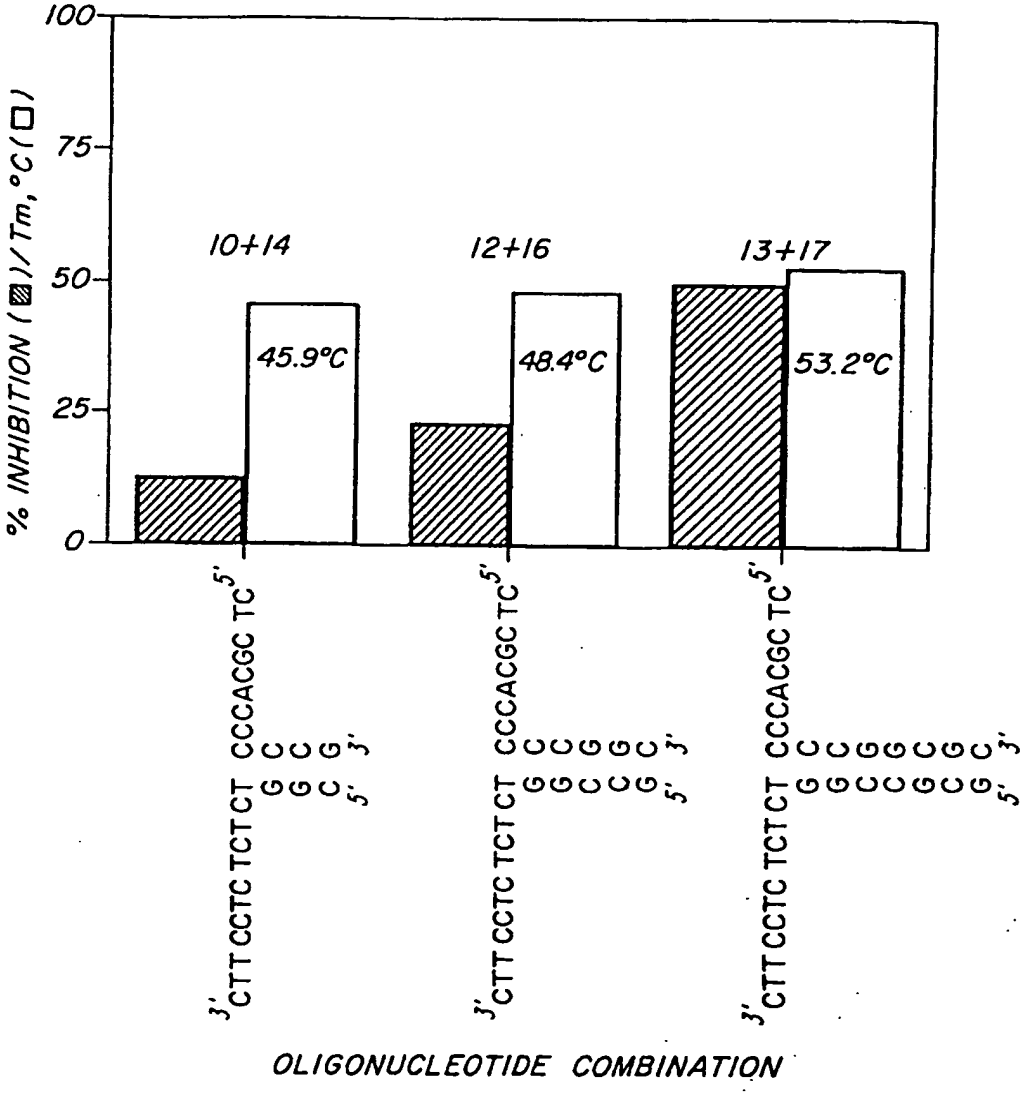
100
75
50
25
0
% INHIBITION (▨)/Tm, °C(□)
10+14
12+16
13+17
45.9°C
48.4°C
53.2°C
3'CTT CCTC TCT CT CCCACGC TC5'
G C
G C C G
5' 3'
3'CTT CCTC TCT CT CCCACGC TC5'
G C C G G C
G G C C G C
5' 3'
3'CTT CCTC TCT CT CCCACGC TC5'
G C C G G C
G G C C G C G
5' 3'
OLIGONUCLEOTIDE COMBINATION

FIG. 8

INTERNATIONAL SEARCH REPORT

Intern ul Application No
PCT/US 97/05683

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 A61K31/70 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RES. (09-1995), 23(17), 3578-84 CODEN: NARHAD;ISSN: 0305-1048, XP002037285 KANDIMALLA, EKAMBAR R. ET AL: "Design, biochemical, biophysical and biological properties of cooperative antisense oligonucleotides" see the whole document ---	1-51
X	WO 95 01985 A (LYNX THERAPEUTICS INC ;GRYAZNOV SERGEI M (US)) 19 January 1995 see page 8, line 28 - page 16, line 14 --- -/--	1-51

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
18 August 1997	29. 08. 97

Name and mailing address of the ISA	Authorized officer
European Patent ffice, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 97/05683

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH, vol. 20, no. 12, 25 June 1992, page 3252 XP000278353 HERTEL K J ET AL: "NUMBERING SYSTEM FOR THE HAMMERHEAD" see the whole document	1-51
Y	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 116, 1994, page 785/786 XP002018814 COLOCCI N ET AL: "COOPERATIVE BINDING OF 8-MER OLIGONUCLEOTIDES CONTAINING 5-(1-PROPYNYL)-2'-DEOXYURIDINE TO ADJACENT DNA SITES BY TRIPLE-HELIX FORMATION" see the whole document	1-51
A	GB 2 225 112 A (ICI PLC) 23 May 1990 see claims 1-13	1-14,32, 36
A	EP 0 185 494 A (APPLIED BIOSYSTEMS) 25 June 1986 see claims 1-17	1,13,32, 36
A	WO 91 06626 A (GILEAD SCIENCES INC) 16 May 1991 see claims 1-12	1,13,32, 36
A	WO 94 17086 A (APOLLON INC ;YOON KYONGGEUN (US); LU MEIQING (US)) 4 August 1994 see figures 9,10	1,13,32, 36
A	WO 94 23028 A (HYBRIDON INC ;AGRAWAL SUDHIR (US); TANG JIN YAN (US); PADMAPRIYA A) 13 October 1994 see claims 1,30; figures 2,3	1,11,42
P,X	WO 96 32474 A (HYBRIDON INC) 17 October 1996 see the whole document	1-51

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INTERNATIONAL SEARCH REPORT

:rnational application No.
PCT/US 97/05683

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: (see below)
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 52-55 completely and 36-51 partially as far as an "in vivo" method is concerned are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compoun/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Intern ...al Application No
PCT/US 97/05683

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9501985 A	19-01-95	US 5571903 A	05-11-96
		AU 7258194 A	06-02-95
GB 2225112 A	23-05-90	DE 3938853 A	23-05-90
		FR 2639358 A	25-05-90
		IT 1238025 B	23-06-93
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		DE 3586090 A	25-06-92
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		JP 61191964 A	26-08-86
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		AU 7148191 A	31-05-91
		EP 0539371 A	05-05-93
		JP 5505101 T	05-08-93
		US 5527899 A	18-06-96
WO 9417086 A	04-08-94	NONE	
WO 9423028 A	13-10-94	AU 6527094 A	24-10-94
		CA 2159350 A	13-10-94
		CN 1124980 A	19-06-96
		EP 0693123 A	24-01-96
		JP 8510900 T	19-11-96
		NZ 263985 A	27-07-97
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